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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: SWA4338P0070US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/089,000

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/01093	22 September 2000 (22.09.00)	22 September 1999 (22.09.99)

TITLE OF INVENTION THREE-DIMENSIONAL STRUCTURE AND CRYSTAL OF A CLASS Iα1,2-MANNOSIDASE, AND METHODS OF USE THEREOF

APPLICANT(S) FOR DO/EO/US Herscovics, Annette; Lipari, Francesco; Sleno, Barry; Howell, Lynne, P. Vallee, Francois; Romero, Pedro, A.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☑ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b. ☑ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☑ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☑ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☑ Other items or information:
    - a. Copy of International Publication Number WP 01/21769 A2 published 29 March 2001;
    - b. Copy of International Search Report;
    - c. Copy of International Preliminary Examination Report; and
    - d. Copy of Form PCT/IB/308.

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U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/089,000

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17. ☑ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO....... $860.00		
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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	-41- - 20 =	-21-	X $18.00	$ 378.00	
Independent claims	- 7- - 3 =	- 4 -	X $80.00	$ 336.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 0.00	

TOTAL OF ABOVE CALCULATIONS =	$ 1,734.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	$ 0.00	
SUBTOTAL =	$ 1,734.00	
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TOTAL NATIONAL FEE =	$ 1,734.00	
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a. ☑ A check in the amount of $ 1,734.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ________ in the amount of $________ to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Date: March 22, 2002

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THREE-DIMENSIONAL STRUCTURE AND CRYSTAL OF A CLASS I α1,2-MANNOSIDASE, AND METHODS OF USE THEREOF

Research in the present invention was supported in whole or in part by a grant from the National Institute of Health of the United States Government under No. GM31265. Hence, the U.S. Government has certain rights in this technology.

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to a three-dimensional structure for endoplasmic reticulum (ER) α-mannosidase, more particularly that of the endoplasmic reticulum α1,2-mannosidase enzyme family, to a crystal and to methods of use thereof.

(b) Description of Prior Art

α-Mannosidases are essential for the maturation of carbohydrate groups on mammalian glycoproteins.

α-Mannosidases have been classified into two distinct groups based on amino acid sequence homology and on biochemical properties. Class I α-mannosidases specifically hydrolyze α1,2-linked mannose residues, and do not cleave substrates such as p-nitrophenyl-α-D-mannopyranoside. They require calcium for activity and are inhibited by 1-deoxymannojirimycin and kifunensine, but not by swainsonine. In contrast, Class II α-mannosidases can cleave α1,2-, α1,3- and α1,6-linked mannose residues as well as p-nitrophenyl-α-D-mannopyranoside and are inhibited by swainsonine, but not by 1-deoxymannojirimycin.

Class I α1,2-mannosidases (family 47 of the glycosyl hydrolase (B. Henrissat, *Biochem. J.* **280**, 309 (1991)) have been conserved throughout eukaryotic evolution for the maturation of N-glycans during glycoprotein biosynthesis (K. W. Moremen, R. B. Trimble, A. Herscovics, *Glycobiology* **4**, 113 (1994);A. Herscovics, *Biochim. Biophys. Acta* **1426**, 275 (1999); A. Herscovics, *Biochim. Biophys. Acta* **1473**, 96 (1999); A. Herscovics, *in Comprehensive Natural Products Chemistry*. B. M. Pinto, Ed. (Elsevier, 1999) **3**, p. 13), but differ in their specificity. N-glycan formation begins with the transfer of a preformed oligosaccharide precursor, usually $Glc_3Man_9GlcNAc_2$, to nascent polypeptide chains. The oligosaccharide precursor is immediately trimmed by α-glucosidases and α-mannosidases in the endoplasmic reticulum (ER). Glycoproteins that have acquired their native conformation can then be transported to the Golgi apparatus, where additional α-mannosidases produce the appropriate substrates for Golgi glycosyltransferases to form the variety of biologically important oligosaccharide structures found on glycoproteins (A. Varki, *Glycobiology* **3**, 97 (1993)).

Besides their importance in N-glycan maturation, ER processing glycosidases also play a role in quality control, ensuring that only properly folded proteins are transported to their final destination. Trimming of the oligosaccharide precursor by α-glucosidase I and II controls the interaction of newly-formed glycoproteins with the lectin chaperones, calnexin and calreticulin, thus facilitating folding of glycoproteins (C.Hammond

and A. Helenius, *Curr. Opin. Cell Biol.* **7**, 523 (1995)), while trimming of mannose residues in the ER acts as a signal to target misfolded glycoproteins for degradation by the proteasome (K. Su, T. Stoller, J. Rocco, J. Zemsky, R. Green, J. *Biol. Chem.* **268**, 14301 (1993); M. Knop, N. Hauser, D. H. Wolf, *Yeast* **12**, 1229 (1996);C. A. Jakob, P. Burda, J. Roth, M. Aebi, *J. Cell Biol.* **142**, 1223 (1998);Y. Liu, P. Choudhury, C. M. Cabral, R. N. Sifers, *J. Biol. Chem.* **272**, 7946 (1997)).

In *Saccharomyces cerevisiae,* there is only one processing α-mannosidase (Swiss Prot accession number P32906). This enzyme is a 63kDa type II ER transmembrane glycoprotein with no significant cytoplasmic tail, an N-terminal transmembrane domain and a large C-terminal catalytic domain (A. Camirand, A. Heysen, B. Grondin, A. Herscovics, *J. Biol. Chem.* **266**, (1991) 15120).

The yeast and human endoplasmic reticulum (ER) α1,2-mannosidases are highly specific and trim $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B, while mammalian Golgi α1,2-mannosidases transform $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$. The yeast α1,2-mannosidase is extremely specific and removes a single mannose residue from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$ isomer B (J. C. Byrd, A. L. Tarentino, F. Maley, P. H. Atkinson, R. B. Trimble, *J. Biol. Chem.* **257**, 14657 (1982); S. Jelinek-Kelly, A. Akiyama, B. Saunier, J. S. Tkacz, A. Herscovics, *J. Biol. Chem.* **260**, 2253 (1985); S. Jelinek-Kelly and A. Herscovics, *J. Biol. Chem.* **263**, 14757 (1988);F. D. Ziegler, T. R. Gemmill, R. B. Trimble, *J. Biol. Chem.* **269**, 12527 (1994)). An ER α1,2-mannosidase with the

same specificity also occurs in mammalian cells(J. Bischoff and R. Kornfeld, *J. Biol. Chem.* **258**, 7907 (1983); J. Bischoff, L. Liscum, R. Kornfeld, *J. Biol. Chem.* **261**, 4774 (1986); L. J. Rizzolo and R. Kornfeld, *J. Biol. Chem.* **263**, 9520 (1988); S. Weng and R. G. Spiro, *J. Biol. Chem.* **268**, 25656 (1993); A. Lal, *et al.*, *Glycobiology* **8**, 981 (1998)). The cDNA encoding the human ER α1,2-mannosidase has recently been cloned (Tremblay, L.O. and Herscovics, A. *Glycobiology* 1999, **9**, 1073). The importance of this enzyme in ER quality control has been demonstrated in both yeast and mammalian cells. In yeast, it was shown that mutant carboxypeptidase Y is stabilized in the *mns1* mutant lacking the ER processing α1,2-mannosidase, while it is rapidly degraded in wild type cells (M. Knop, N. Hauser, D. H. Wolf, *Yeast* **12**, 1229 (1996); C. A. Jakob, P. Burda, J. Roth, M. Aebi, *J. Cell Biol.* **142**, 1223 (1998)). In mammalian cells, the ER degradation of foreign (K. Su, T. Stoller, J. Rocco, J. Zemsky, R. Green, *J. Biol. Chem.* **268**, 14301 (1993)) or abnormal glycoproteins such as mutant α1-antitrypsin (Y. Liu, P. Choudhury, C. M. Cabral, R. N. Sifers, *J. Biol. Chem.* **274**, 5861 (1999)), is prevented by the α1,2-mannosidase inhibitor, 1-deoxymannojirimycin. This processing α1,2-mannosidase may therefore have an important role in genetic diseases characterized by rapid degradation of misfolded glycoproteins such as cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis and α1-antitrypsin in emphysema (R. N. Sifers, *Nat. Struct. Biol.* **2**, 355 (1995)).

The action of the ER α1,2-mannosidases in both yeast and mammalian cells triggers the degradation of misfolded glycoproteins. When the action of the ER α1,2-mannosidase is inhibited, misfolded glycoproteins are considerably more stable, are not as easily degraded and are secreted (Marcus, N. Y. and Perlmutter, D. H. *J. Biol. Chem.* **275**, 1987 (2000)).

It is possible to identify inhibitors of the ER α1,2-mannosidases by screening a large number of natural and synthetic compounds. However, it would be advantageous to screen drugs based on a determined three-dimensional enzyme-inhibitor complex, and to design potential antagonists using computer modeling.

The three-dimensional structures of the Class I α1,2-mannosidases remain at present unknown, however, X-ray crystallographic data has been obtained.

It would therefore be highly desirable to be provided with a three-dimensional structure for the endoplasmic reticulum (ER) α1,2-mannosidase enzyme family.

It would also be highly desirable to be provided with a crystallized form of the enzyme, to allow X-ray crystallographic data to be obtained.

This would allow the identification of structural determinants responsible for the specificity of the different enzymes of the α1,2-mannosidase enzyme family. In addition, a crystal structure of an α1,2-mannosidase enzyme could be used in determining homologous enzyme structures in other species.

This would also be useful for developing drugs such as inhibitors specific to the ER α1,2-mannosidase

to stabilize abnormal, misfolded glycoproteins in genetic diseases, such as mutant CFTR in cystic fibrosis and α-antitrypsin in pulmonary emphysema and glycoproteins in any other genetic diseases characterized by rapid degradation of misfolded glycoproteins.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide three-dimensional structures and crystals for endoplasmic reticulum (ER) α1,2-mannosidase enzymes, and more particularly detailed three-dimensional structural information for the ER α1,2-mannosidase enzyme family.

Another aim of the present invention is to provide identification of structural determinants responsible for the specificity of the different enzymes of the α1,2-mannosidase enzyme family.

Yet another aim of the present invention is to provide methods to develop drugs such as agonist, antagonist or inhibitors specific for the ER α1,2-mannosidase enzyme family, which may be used to develop drugs to stabilize abnormal, misfolded glycoproteins in genetic diseases, including, without limitations, cystic fibrosis and pulmonary emphysema. For example, the agonist or antagonist may activate or inhibit the activity of the enzyme for a transient period of time, preventing or activating degradation of misfolded, abnormal glycoproteins.

The yeast ER α1,2-mannosidase is the first member of the Class I α1,2-mannosidases whose three-

dimensional structure has been determined. The recombinant enzyme was purified, crystallized and the structure was established by X-ray crystallographic techniques using the single isomorphous replacement with anomalous scattering (SIRAS) method. The structure obtained consists of a novel $\alpha\alpha_7$ fold. Protein-carbohydrate interactions within the active site, or catalytic domain, are visualized and the interactions responsible for differences in enzyme specificity may be identified.

The three-dimensional structure of the yeast α1,2-mannosidase of the present invention may be used to deduce that of the enzyme of other species, such as mammalians, and more particularly the human.

The three-dimensional structure of the yeast α1,2-mannosidase of the present invention may also be used to deduce that of other members of this enzyme family and to develop specific inhibitors that may be used to stabilize abnormal, misfolded glycoproteins and render them more functional. Likewise, alterations in the nucleic acid sequence of the α1,2-mannosidase of the present invention may result in an enzyme with a novel specificity.

Based on the three-dimensional structures provided herein, drugs may be developed to control genetic diseases caused by glycoprotein misfolding including cystic fibrosis and emphysema, such as by computer analyses with a computer program that analyzes molecular structure and interactions.

In accordance with the present invention, there is provided a crystal of a protein-

oligosaccharide/carbohydrate complex comprising a catalytic domain of a class I α1,2-mannosidase enzyme trimming $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B, said crystal effectively diffracting X-rays at a resolution of about 1.54 Angströms, thus providing the first detailed three-dimensional structure of a Class I α1,2-mannosidase.

The class I α1,2-mannosidase enzyme may more particularly consist of the endoplasmic reticulum (ER) class I α1,2-mannosidase enzyme.

The α1,2-mannosidase may be derived from a yeast such as *Saccharomyces cerevisiae*. The α1,2-mannosidase may also be derived from a mammalian, and more particularly a human.

The catalytic domain may comprise a space group of $P3_121$ and a unit cell of dimensions of $\alpha=\beta=90.00$ and $\gamma=154.6$. The catalytic domain may also comprise a barrel of seven pairs of helices $(\alpha\alpha_7)$, and the pairs of helices may consist of a first set of parallel helices inner-disposed in the barrel, and a second set of helices anti-parallel to the first set.

In accordance with the present invention, there is provided a method for determining a three-dimensional structure of an α1,2-mannosidase, which comprises using a three-dimensional structure of α1,2-mannosidase of a yeast to derive a α1,2-mannosidase three-dimensional structure of another species therefrom.

The deriving may be effected by molecular replacement.

The three-dimensional structure may be from a mammalian, and more particularly a human.

The first three-dimensional structure of the yeast α1,2-mannosidase may be a member of the class I α1,2-mannosidase family.

In accordance with the present invention, there is also provided a method of using such a crystal in a drug screening assay. The method comprises selecting a potential antagonist or inhibitor by performing rational drug design with the three-dimensional structure determined for the crystal, the selecting being performed in conjunction with computer modeling, adding the potential antagonist or inhibitor to a glycoprotein synthesis assay in which the α1,2-mannosidase is a rate-limiting factor, and detecting a change of protein synthesis, wherein a potential antagonist or inhibitor that inhibits maturation of carbohydrate on a newly formed glycoprotein and stabilizes a misfolded glycoprotein is selected as a potential drug.

Drugs may be screened for a specific inhibitor or antagonist of an ER α1,2-mannosidase enzyme, to stabilize a misfolded glycoprotein in a genetic disease. The disease may consist of cystic fibrosis or pulmonary emphysema, and the glycoprotein may consist of a mutant of cystic fibrosis transmembrane conductance regulator (CFTR) or α1-antitrypsin, respectively.

This three-dimensional structure of the Class I processing α1,2-mannosidase of the present invention provides a framework to understand the mechanism of

action, to determine the basis of the differences in specificity of the different family members and to elucidate their respective roles in glycoprotein maturation.

In accordance with the present invention, there is provided a expression vector comprising the nucleic acid of SEQ ID NO:1 operatively associated with an expression control sequence

In addition, mutant forms of the ER α1,2-mannosidase enzyme have been determined to have altered specificity.

The knowledge of the structure of the active site of the yeast enzyme provided herein and the mode of substrate-binding may be used to develop specific inhibitors for preventing the degradation of abnormal, misfolded glycoproteins caracteristic of genetic diseases including cystic fibrosis and emphysema.

The expression "active site cavity" is intended to mean the active site within the barrel which is the region where the amino acid residues essential for catalysis and the essential calcium ion are located; i.e. it is where the action of the enzyme occurs during catalysis, where cleavage of mannose from the substrate occurs.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the schematic ribbon representation of the three-dimensional structure of the yeast α1,2-mannosidase viewed down the $\alpha\alpha_7$ barrel axis;

Fig. 2 illustrates the ribbon representation at 90° to the first orientation, and the protein-protein interaction in the crystal packing;

Fig. 3 illustrates a schematic representation of the high-mannose oligosaccharide HM1;

Fig. 4 illustrates a detailed high-mannose oligosaccharide (HM)-enzyme interaction between HM1 and the protein;

Fig. 5 illustrates a Van der Waals surface representation of the high-mannose oligosaccharide (HM)-enzyme interaction; and

Fig. 6 illustrates the electrostatic surface of the yeast α1,2-mannosidase, showing the size of the catalytic groove and the location of the high-mannose oligosaccharide HM1.

Fig. 7 illustrates the interactions between the yeast ER α1,2-mannosidase, calcium and 1-deoxymannojirimycin.

Fig. 8A at the top illustrates the order of removal of mannose from $Man_9GlcNAc_2$ by the R273L mutated form of the ER α1,2-mannosidase enzyme of the present invention.

Fig. 8B illustrates the effect of a single point mutation of arginine 273 to leucine on the specificity of the α1,2-mannosidase enzyme of the present invention. The time course of formation of products from $Man_9GlcNAc$ substrate by the mutant in Fig. 8A is compared to the non-mutated ER α1,2-mannosidase shown in Fig. 8B.

Fig. 9 illustrates the amino acid sequence in capital letters and the corresponding nucleotide

sequence of the R273L yeast ER α1,2-mannosidase mutant. The complete open reading frame is shown, the arrow indicates the beginning of the catalytic domain and the mutated residue is boxed.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a crystal of a class I α1,2-mannosidase. The crystal structure of the catalytic domain, or active site, of the endoplasmic reticulum (ER) yeast α1,2-mannosidase that transforms $Man_9GlcNAc_2$ to a single isomer of $Man_8GlcNAc_2$ (isomer B lacking the α1,2-linked mannose on the α1,3 mannose of the α1,6 branch) was determined at 1.5 Angström resolution. This enzyme functions in ER quality control and triggers the mechanisms leading to degradation of misfolded glycoproteins in certain genetic diseases including mutants of CFTR in cystic fibrosis and α1-antitrypsin in emphysema.

The Class I α1,2-mannosidases of the present invention differentiate from Class II α-mannosidases with respect to their structure, enzymatic properties and catalytic mechanism.

This is the first member of this enzyme family for which a three-dimensional structure has been determined. The three-dimensional structure of this enzyme and of its active site may be used to design specific inhibitors that may stabilize misfolded glycoproteins in diseases. The three-dimensional structure provided herein may also be used to determine

the three-dimensional structure of other members of this enzyme family.

The crystal structure of the endoplasmic reticulum enzyme of *Saccharomyces cerevisiae* reveals a novel $\alpha\alpha_7$ barrel in which one of the N-glycans from one molecule extends into the barrel of the adjacent molecule interacting with the essential acidic residues and calcium ion. The observed protein-carbohydrate interactions provide a first insight into the catalytic mechanism and specificity of this eukaryotic enzyme family and may be used to develop inhibitors that prevent degradation of misfolded glycoproteins in genetic diseases.

The catalytic domain of the yeast α1,2-mannosidase was produced in *P. pastoris* as a secreted glycoprotein (F. Lipari, A. Herscovics, *Glycobiology* **4**, 697 (1994); F. Lipari, B. J. Gour-Salin, A. Herscovics, *Biochem. Biophys. Res. Commun.* **209**, 322 (1995); F. Lipari and A. Herscovics, J. *Biol. Chem.* **271**, 27615 (1996)). The α1,2-mannosidase nucleotide sequence encoding amino acids 34-549 from the known sequence was amplified by PCR and ligated into the XhoI and BamHI sites of the pHIL-S1 vector (Invitrogen) producing plasmid YpHΔ33. The nucleotide sequence encoding amino acids 367-371 in the plasmid YpHΔ33 was then deleted using the U.S.E Mutagenesis kit from Pharmacia Biotech Inc. The protein was expressed in *P. pastoris* and purified.

Crystals were grown by the vapor diffusion method from protein drops (10 mg/ml) equilibrated against well solution (1 ml) containing 17-19%

polyethylene glycol 2K MME, 100 mM sodium citrate (pH 5.6) and 250 mM ammonium acetate. Crystals grew within 5 days and exhibit the symmetry of space group $P3_121$ and a unit cell of dimensions of $\alpha=\beta=90.00$ Å and $\gamma=154.6$ Å), with one molecule in the asymmetric unit (59% solvent content).

Native and derivative data were first collected at room temperature using monochromated CuKα X-radiation (Rigaku Rotaflex RU200 rotating anode generator) on a Mar Research (345 mm diameter) imaging plate system. Subsequent native data sets were collected on beamline X8C at the N.S.L.S. (Brookhaven National Laboratory, Upton, N-Y, U.S.A) using a Quantum4 CCD detector and flash-frozen crystals (cryoprotected in artificial mother liquor containing 25% v/v glycerol). All data were processed with DENZO™ and SCALEPACK™.

The structure was determined using the single isomorphous replacement with anomalous scattering (SIRAS) technique. This is the first processing enzyme in N-glycan biosynthesis whose three-dimensional structure has been determined. The structure has been refined to a R_{crist} of 21.2% and a R_{free} of 22.8% for the data between 50 and 1.54 Å resolution, as may be seen in Table 1. The structure of the yeast ER α1,2-mannosidase catalytic domain complexed with the inhibitor 1-deoxymannojirimycin was also determined at 1.59 Angströms resolution. This structure has been refined to a R_{cryst} and R_{free} of 21.6% and 24.4%, respectively.

Table 1

Three-dimensional structure determination

Diffraction data	Native 1	$HgCl_2$	Native 2	Native 3
X radiation (λ, Å)	Rigaku RU200 (1.54)	Rigaku RU200 (1.54)	NSLS X8-C (1.00)	NSLS X8-C (0.975)
Resolution (Å)	2.71	2.71	2.00	1.54
Unit Cell (a, b, c; Å)*	90.04 90.04 154.87	90.07 90.07 154.61	88.83 88.83 153.61	88.38 88.38 153.32
Temperature (°C)	20	20	-180	-180
Measured Reflections	90034	88356	350472	649016
Unique reflections	20423	20049	45749	100985
Redundancy	4.5	4.4	9	6.5
Completeness'	99 (99)	99 (99)	96.4 (98.4)	99.5 (99.3)
R_{sym}'	0.071 (0.14)	0.081 (0.16)	0.058 (0.27)	0.062 (0.44)
R_{deriv}'		0.141		
Sites (n)		1		
R_{Cullis}'		0.52		
Phasing power		1.63		
F.O.M. before (after) Solvent flattening		0.42 (0.84)		

Refinement statistics			
Resolution (Å)	50-1.54	Rmsd bond length (Å)	0.005
R_{cryst}'	21.2	Rmsd bond angles	1.2
R_{free}'	22.8	rmsd B values $(Å)^2$	2.1

*Space group $P3_121$; $\alpha=\beta=90°$ and $\gamma=120°$;

Given in parentheses are the completeness' and R_{sym}' for the last resolution shell.

The three-dimensional structure was determined by the SIRAS phasing method using a single site mercury derivative. The program C.N.S. was used for all stages of the structure determination and refinement as described in Crystallographic programs and methods. C.N.S.: A. T. Brunger, *et al.*, *Acta Crystallogr.* **D54**, 905 (1998); TURBO-FRODO: A. Roussel and C. Cambillau, Silicon Graphics Geometry Directory 86 (Silicon Graphics, Mountain View, CA, (1992); PROCHECK: R. A. Laskowski, M. W. MacArthur, D. S. Moss, J. M. Thornton, *J.Appl. Crystallogr.* **26**, *X* (1993); DENZO and SCALEPACK: Z. Otwinowski, W. Minor, *Methods Enzymol.* **276**, *X* (1997); MOLSCRIPT: J. Kraulis, *J.Appl. Cryst.* **24**, 946 (199 1); RASTER3D: E. A. Merritt and M. E. P. Murphy, *Acta Crystallogr.* **D50**, 869 (1994); GRASP: A. Nicholls, K. A. Sharp, B. Honig, *Proteins* **6**, 281 (1993), which is herein incorporated by reference.

The atomic position of the Hg atom was found using Patterson methods. The Hg coordinates were refined and the phases calculated were subsequently improved by solvent flattening, improving the mean figure of merit (FOM) from 0.42 to 0.84. The initial solvent-flattened SIRAS map showed a monomeric protein well-separated from the solvent. Interpretable regions of the map were used to build strands of polyalanine chains with the program TURBO-FRODO™. The stepwise recombination of the phases from the growing polyalanine model with the original SIRAS phases continuously improved the FOM and enhanced the quality of the electron density map. When approximately 82% of the protein backbone positions had been traced, the side chains were inserted using the one disulfide

bridge clearly identifiable in the electron density, and the three N-glycosylation sites as starting points for fitting the sequence. The model was completed by monitoring the R_{free} of the model and the FOM of the combined model/SIRAS phases and then refined using the simulated annealing slow-cooling protocol in the resolution range of 50 to 1.54 Å. All residues have been modeled with the exception of residue 410 owing to weak electron density in this region.

The model comprises 4117 non-hydrogen protein atoms, together with 414 water molecules, 135 carbohydrates atoms, 6 glycerol atoms plus a Ca^{2+} ion. In the final structure, none of the non-glycine residues lie in the disallowed region of the Ramachandran plot as analyzed with PROCHECK™.

The equations used are as follows:

$$R_{cryst} = \Sigma \left| \, |F_o| - |F_c| \, \right| / \Sigma \, F_o$$

where F_o and F_c are the observed and calculated structure factors, respectively;

$$R_{sym} = \Sigma\Sigma \left| \, I_i - <I> \right| \Sigma \, I_i$$

where <I> is the average of equivalent reflections and the sum is extended over all measured observations for all unique reflections;

$$R_{deriv} = \Sigma \left\| \, F_{PH} \right| - |F_P| \| / \Sigma \, |Fp| ;$$

$$R_{Cullis} = \Sigma \left\| \, F_{PH} + F_P \, \right| - | \, F_H(calc) \, \| / \Sigma \, | \, F_{PH} - F_P |$$

for centric reflections;

For R_{free}, the sum is extended over a subset of reflections (10%) excluded from all stages of refinement (9536 reflections).

Phasing power, root mean square (rms) F_H / rms ε where ε is lack of closure and F_H is the calculated heavy atom structure factor.

The final model contains residues 34 to 367, 371 to 409 and 411 to 549 as well as 414 solvent molecules, one glycerol molecule, one calcium ion and three N-glycans.

Referring now to Fig. 1, the calcium ion (Ca) is represented as a sphere, and the glycerol molecule (G), the three high-mannose oligosaccharides (HM1, HM2 and HM3), the ASN residues attached to these oligosaccharides and the two disulfide bridges (S1: Cys340-Cys385 and S2: Cys468-Cys471) in ball-and-stick representation. Referring more particularly, to Fig. 2, HM1 extends into the barrel of the adjacent molecule, thus facilitating the crystallization. Located in the protein-protein interface, the reconstructed loop (RL) is also important for the crystallization.

As can be seen in Figs 1 and 2, the α1,2-mannosidase catalytic domain is an $\alpha\alpha_7$ helix barrel with overall dimensions of approximately 50 Å x 50 Å x 50 Å. This is the first example of an $\alpha\alpha$-helix barrel consisting of seven pairs of helices. The molecule consists of consecutive helices alternating from outside to inside the barrel. This results in a topology of seven parallel inner helices (α2, α4, α6, α8, α10, α12, α14) and a second set of seven parallel outer helices (α1, α3, α5, α7, α9, α11, α13) concentric to the inner helices and anti-parallel to them. The structure is stabilized by a disulfide, Cys340-Cys385,

which forms a first bridge between the inner α10 helix and the outer α11 helix. This disulfide bond occurs between residues conserved in all known members of the family and was shown previously to be essential for enzyme activity (F. Lipari and A. Herscovics, *J. Biol. Chem.* **271**, 27615 (1996)). A second disulfide bond (Cys468-Cys471) located in a loop between outer α13 and inner α14 helices is unlikely to be important, since these residues are not conserved across the superfamily and mutation of these cysteines did not greatly affect enzyme activity.

As may be seen in Fig. 2, the two ends of the barrel, the SC and the LC side, are structurally distinct. On the SC side, the pairs of inner and outer helices are connected by short loops of up to 4 residues, with the exception of a loop consisting of 10 residues that links the α10 and α11 helices. Three high-mannose oligosaccharides (HMI, HM2 and HM3) are found at the predicted N-glycosylation sites. These oligosaccharides extend away from the surface of the protein on the SC side of the barrel. Only one and three sugar residues of HM2 and HM3, respectively, were found in the electron density, due to the flexibility of the oligosaccharides and their lack of interaction with the protein in the crystal. In contrast, five mannose and two N-acetyl glucosamine residues were clearly identified in HM1, which behaves as a substrate/product of the enzyme, as described hereinafter.

The LC side is structurally more complex and is similar to an "open flower" with strands forming the petals of the flower. The ß-strands pack together to form a series of anti-parallel ß-sheets surrounding the helix-barrel. The C-terminal of the protein (residues 512-549) consists of a ß-hairpin protruding back into the center of the barrel from the SC side and an additional short helix, α15.

The ß-hairpin blocks the barrel, preventing the protein from looking like an open channel. The ß-hairpin, the inner helices and the ß-sheets on the LC side of the barrel result in a cavity of approximately 15 Å in depth, parallel to the central axis of the barrel, with a diameter of 25 Å at the level of the ß-sheets decreasing to approximately 10 Å at the top of the ß-hairpin. This cavity is a consequence of the seven pairs of helices present in the barrel, as no significant cavity is found in $\alpha\alpha_6$-barrel proteins (A. Aleshin, A. Golubev, L. M. Firsov, R. B. Honzatko, J *Biol. Chem.* **267**, 19291 (1992); P.M. Alzari, H. Souchon, R. Dominguez, *Structure* **4**, 265 (1996); H.W. Park, S.R. Boduluri, J.F. Moomaw, P.J. Casey, L.S. Beese, *Science* **275**, 1800 (1997); B. Nagar, R.G. Jones, R.J. Diefenbach, D.E. Isenman, J.M. Rini, *Science* **280**, 1277; G. Parsiegla, M. Juy, C. Reverbel-Leroy, C. Tardif, J-P. Belaich, H. Driguez, R. Haser, *EMBO J.* **17**, 19, 5551 (1998)). The nine highly conserved acidic residues and the calcium ion, all of which are essential for catalytic activity (F. Lipari and A. Herscovics, *Biochemistry* **38**, 1111 (1999)) are located at the top of this ß-hairpin in the center of the $\alpha\alpha_7$-helix barrel,

indicating that this region contains the active site of the enzyme.

Referring to Fig. 3, the α1,2 linkage between mannose M7 and MIO of HM1 is specifically cleaved by the enzyme, yielding $Man_8GlcNAC_2$ isomer B, the smallest oligosaccharide found in yeast N-glycans. Referring to Fig. 5, the residues as well as the glycerol molecule and the calcium ion are completely buried in the active site. E132 and D275, the putative catalytic residues, are located at the entrance of a cavity. The insert shows the position of the glycerol molecule, thought to mimic the oligosaccharide residue M1O.

A remarkable interaction is observed between adjacent molecules in the crystal, allowing a more detailed characterization of the active site. Returning to Fig. 3, the HM1 oligosaccharide from one molecule extends into the barrel of the adjacent symmetry-related molecule with the visible terminal mannose residues located in the cavity containing the essential acidic residues and the calcium ion required for activity, as shown in Figs. 4 and 5. This protein-oligosaccharide interaction corresponds to the enzyme-product complex, since the mannose residue of the substrate has been cleaved.

The HM1-protein interaction produces approximately 47% of the overall intermolecular contacts found in the crystal packing, and it is interesting to note that no crystals were obtained from the N96Q mutant lacking HM1, indicating that the interaction facilitated crystallization.

Analysis of the HM1-protein contacts shows that a large proportion of charged residues are involved in carbohydrate stabilization *via* either H-bonds or van der Waals interactions. At the top of the catalytic cavity, the HM1 core residues, NAG2 and M3, only contact R273, as shown in Figs. 4, 5 and 6. R269 and R273 are two arginine residues which disrupt the overall electronegative surface of the catalytic groove. Although not visible in the electron density maps, the surface clearly indicates where the additional oligosaccharide residues M6, M9 and M11 could be located in the active site (see arrows in Fig. 6). The contour level is at +/-20 kT.

The α1,6-branched residue M4 contacts R273 and R433, and M6 contacts R269, S272, D336, L338 and E399, whereas residue M5 of the α1,3-branch, contacts residues S184, S185, N129 and N196. When considering the specificity of the yeast α1,2-mannosidase, M7, that would form the target glycosidic bond with M10 in the substrate, is located towards the bottom of the catalytic site and binds to residues F131, E207, R273 and D275.

Of the residue that interacts with HM1, the only residues which are conserved in all members of family 47 of the glycosyl-hydrolases are D275 and R433 whereas R269, S272, N129 and N196 are specific for the yeast α1,2-mannosidase (R. N. Sifers, *Nat. Struct. Biol.* **2**, 355 (1195)). R273 is only found in the yeast and human class I ER α1,2-mannosidases known to specifically form the $Man_8GlcNAc_2$ B isomer. R273 forms hydrogen-bonds through its Nε, Nη1 and Nη2 atoms with M3, M4, M7 and

NAG2. R273 may therefore together with R269, S272 and D336 stabilize the α1-6 arm of HM1 and could potentially dictate the conformation of the oligosaccharide required by the enzyme to cleave the glycosidic linkage between M7 and M10.

With the three-dimensional structure of the yeast ER α1,2-mannosidase now determined according to the present invention, this structure can be used to determine the structure of the corresponding human α1,2-mannosidase. For example, molecular replacement can be employed, based on the yeast ER α1,2-mannosidase structure, to determine the human α1,2-mannosidase structure.

From the determined protein structures, the specificity of the ER α1,2-mannosidase was changed by site-directed mutagenesis of a single amino acid residue that was seen to interact with the oligosaccharide in the crystal structure. Particularly, arginine[273] was replaced by leucine and the specificity of the α1,2-mannosidase produced in *Pichia pastoris* was altered.

The R273L mutant was found to remove additional mannose residues from $Man_9GlcNAc$ with $Man_5GlcNAc$ as the end product (Fig. 8A). In contrast, primarily $Man_8GlcNAc$ is formed by the parent enzyme (Fig. 8B). The oligosaccharides formed from $[^3H]Man_9GlcNAc$ were fractionated by HPLC, and the time course of oligosaccharide product formation was compared with that obtained for the parent enzyme (Fig.8B). The time course of trimming $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ by the R273L mutant is shown in Fig. 8A compared to the time

course of trimming of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ by the wild type ER α1,2-mannosidase shown in Fig. 8B. High resolution ^{1}H-NMR of the oligosaccharide intermediates formed by the R273L mutant showed that the order of mannose removal from $Man_9GlcNAc_2$, shown at the top of Fig. 8, is different from that previously observed for mammalian Golgi α1,2-mannosidases. Therefore, a single mutation in the catalytic domain of the yeast ER α1,2-mannosidase produces an enzyme with novel specificity. Expression of the mutant ER α1,2-mannosidase intracellularly should modify the mannose trimming in the endoplasmic reticulum and may affect the fate of misfolded glycoproteins.

The yeast α1,2-mannosidase is an inverting glycosyl-hydrolase (F. Lipari, B. J. Gour-Salin, A. Herscovics, *Biochem. Biophys. Res. Commun.* **209**, 322 (1995)). The catalytic mechanism involves two acidic residues, one acting as a base removing a proton from water and the other acting as an acid donating a proton to the leaving group. Among the invariant acidic residues found in Class I α1-2 mannosidases, E132 is likely to be the catalytic base as it is the only acidic residue to interact indirectly *via* a water molecule ($d_{E132-WAT}$=2.74 Å) with the glycosidic linkage to be cleaved ($d_{WAT-SACC}$=3.40 Å). This hypothesis is supported by kinetic data showing a decrease in k_{cat}, a change typical of glycosidases with mutations in their catalytic residues (F. Lipari and A. Herscovics, *Biochemistry* **38**, 1111 (1999)). In inverting enzymes, the average distance between the four acidic oxygen atoms of the acid and the base is between 6.5 and 9.5 Å

(J. D. McCarter and S. G. Withers, *Curr. Opin. Struct. Biol.* **4**, 885 (1994)). This geometrical constraint as well as the spatial disposition of both residues relative to the glycosidic linkage to be cleaved suggest that D275 is the probable proton donor since it is the only essential acidic residue located on the opposite side of M7 about 8.5 Å from E132. This hypothesis is consistent with the complete lack of activity of the D275N mutant (F. Lipari and A. Herscovics, *Biochemistry* **38**, 1111 (1999)).

The agreement between the crystal structure described herein and the previously reported site-directed mutagenesis experiments (F. Lipari and A. Herscovics, *Biochemistry* **38**, 1111 (1999)) clearly indicates that the protein-carbohydrate interactions observed in the crystal packing are biologically relevant. As seen in Fig. 6, the close complimentarity found between protein and carbohydrate surfaces also supports this hypothesis. Additional evidence is provided by the position of a glycerol molecule introduced during crystal freezing. Glycerol has been shown previously to mimic saccharide binding (A. Schmidt, A. Schlacher, W. Steiner, H. Schwab, C. Kratky, *Protein Sci.* **7**, 2081 (1998)).

In the structure of the present invention, a glycerol molecule is observed at the bottom of the active site cleft close to M7 and the calcium ion, suggesting that it occupies the putative binding site for M10, as shown in Figs. 4 and 5. The three oxygen atoms of the glycerol molecule hydrogen bond to glutamic acid (E435, E438, E503) and arginine (R433) residues. Glycerol also forms van der Waals

interactions with F499 at the bottom of the catalytic cavity, suggesting that this strictly conserved residue is involved in substrate stabilization.

Calcium has been shown to play an important role in enzyme activity. The calcium ion binds to the carbonyl oxygen and the Oγ of T525 located at the top of the ß-hairpin and to four water molecules which are in turn H-bonded to one of the two carboxylate groups of residues E279, E435, E438 and E503, previously demonstrated as crucial for enzymatic activity (F. Lipari and A. Herscovics, *Biochemistry* **38**, 1111 (1999)), as shown in Fig. 4.

The three-dimensional structure of the yeast ER α1,2-mannosidase complexed with the inhibitor 1-deoxymannojirimycin was determined by X-ray crystallography.

Table 2

Data collection and refinement statistics for the mannosidase inhibitor complex

Diffraction data	**dMNJ**
X-radiation (λ in Å)	NSLS, X8C (1.00)
Resolution (Å)	1.59
Cell dimensions (a,b,c (Å))[a]	89, 89, 153.1
Temperature (°C)	-160
Measured reflections	549334
Unique reflections	95073
Completeness[b]	99.2 (96.5)
Average $I/\sigma(I)$[b]	9.0
R_{sym}[b]	0.07 (0.44)
Refinement statistics	
Resolution (Å)	50-1.59
No. Protein/Solvent atoms	567
No. inhibitor atoms	11
R_{cryst}	0.213
R_{free}	0.238
Rmsd bond length (Å)	0.006
Rmsd bond angles	1.3
Rmsd B values (Å^2)	21.7
Mean B Value (Å^2)	
Protein : Overall B value (Main chain / Side chain)	26.6 (25.9 / 27.4)
Solvent	40.6
Calcium	21.7
Inhibitor	26.4

[a] Space Group $P3_121$; $\alpha = \beta = 90°$, $\gamma = 120°$.

The inhibitor is found at the top of the C-terminal β-hairpin at the bottom of the active site cavity. The 2′ and 3′ hydroxyl groups of the inhibitor ring are involved in coordination of the calcium ion (Fig. 7). Inhibitor binding stabilizes the calcium ion which is 8-fold coordinated with a pentagonal bi-pyramidal geometry. One of the apices of the pyramid is occupied by the O2′ and O3′ hydroxyl groups of the inhibitor. In the absence of inhibitor two water molecules are coordinated to the calcium ion. Hydroxyl groups of 1-deoxymannojirimycin form hydrogen bond with residues R433, E435, E503, E526 and *via* water molecules to residues E132, E438, E279 and D275 (Fig. 7). These conserved acidic residues at the bottom of the active site cavity have been shown by mutagenesis to play an important role in catalysis.

The O6′ hydroxyl forms hydrogen bonds with E435 and with R433. No large global conformational change was observed upon inhibitor binding, but there was a small change in the position of the R433 side chain upon inhibitor binding. The six-membered ring of 1-deoxymannojirimycin has a non-standard deformed $^{1}C_{4}$ conformation when bound to the enzyme. The position of the inhibitor mimics the location of the mannose residue that would be cleaved during catalysis and suggests that the C1 atom of 1-deoxymannojirimycin corresponds to the C1 position of M10 mannose in the substrate.

The enzyme-inhibitor complex provides an understanding of the catalytic mechanism of the α1,2-mannosidase, the role of calcium and the role of the

acidic residues conserved in all members of the Class I $\alpha 1,2$-mannosidase enzyme family that were shown to be required for enzyme activity. It also provides information to be used for rational drug design using computer modelling.

Based on the inhibitor complex of the present invention, other inhibitors of $\alpha 1,2$-mannosidase can now be determined and modeled.

From the determined protein structures, the specificity of the ER $\alpha 1,2$-mannosidase was changed by site-directed mutagenesis of a single amino acid residue that was found to interact with the oligosaccharide. Particularly, Arginine^{273} was replaced by leucine and the specificity of the $\alpha 1,2$-mannosidase was altered (SEQ ID. NO:1).

The R273L mutant was found to remove additional mannose residues from $Man_9GlcNAc$ with $Man_5GlcNAc$ as the end product (Fig. 8A). In contrast, primarily $Man_8GlcNAc$ is formed by the parent enzyme. When arginine^{273} was mutated to leucine the specificity of the R273L mutant enzyme produced in *Pichia pastoris* was altered. The oligosaccharides formed from $[^3H]Man_9GlcNAc$ were fractionated by HPLC, and the time course of oligosaccharide product formation was compared with that obtained for the parent enzyme (Fig. 8B). The time course of trimming $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ by the R273L mutant is shown in graph A compared to the time course of trimming of $Man_9GlcNAc_2$ to $Man_8GlcNAC_2$ by the wild type ER $\alpha 1,2$-mannosidase shown in graph B of Fig. 8B. High resolution ^1H-NMR of the oligosaccharide

intermediates formed by the R273L mutant showed that the order of mannose removal from $Man_9GlcNAc_2$, shown in Fig. 8A, is different from that previously observed for mammalian Golgi α1,2-mannosidases. Therefore, a single mutation in the catalytic domain of the yeast ER α1,2-mannosidase produces an enzyme with novel specificity. Expression of the mutant ER α1,2-mannosidase intracellularly should modify the mannose trimming in the endoplasmic reticulum and may affect the fate of misfolded glycoproteins.

The three-dimensional structure of this mutant form of the ER α1,2-mannosidase may be determined on the basis of the known structure of the ER α1,2-mannosidase of the present invention.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A crystal of a protein-carbohydrate complex comprising the catalytic domain of a class I α1,2-mannosidase enzyme, said α1,2-mannosidase specifically converting $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B of a glycoprotein, said crystal effectively diffracting X-rays at a resolution of about 1.54 Angströms.

2. A crystal according to claim 1, wherein said class I α1,2-mannosidase enzyme consists of the endoplasmic reticulum class I α1,2-mannosidase enzyme.

3. A crystal according to claim 2, wherein said α1,2-mannosidase is derived from a yeast.

4. A crystal according to claim 3, wherein said yeast consists of *Saccharomyces cerevisiae*.

5. A crystal according to claim 4, wherein said catalytic domain comprises a space group of $P3_121$ and a unit cell of dimensions of $\alpha=\beta=90.00$ and $\gamma=154.6$.

6. A crystal according to claim 5, wherein said catalytic domain comprises a barrel of seven pairs of helices $(\alpha\alpha_7)$.

7. A crystal according to claim 6, wherein said pairs of helices consist of a first set of parallel

helices inner-disposed in the barrel, and a second set of helices anti-parallel to the first set.

8. A method for determining a three-dimensional structure of an α1,2-mannosidase, which comprises using a three-dimensional structure of α1,2-mannosidase of a yeast to derive a α1,2-mannosidase three-dimensional structure of another species therefrom.

9. A method according to claim 8, wherein the deriving is effected by molecular replacement.

10. A method according to claim 9, wherein the first three-dimensional structure is from a mammalian.

11. A method according to claim 10, wherein the mammalian is a human.

12. A method according to claim 9, wherein the first three-dimensional structure of the yeast α1,2-mannosidase is a member of the class I α1,2-mannosidase family.

13. A method of using a crystal according to claim 1 in a drug screening assay, the method comprising:

a) selecting a potential antagonist or inhibitor by performing rational drug design with the three-dimensional structure determined for a crystal according to claim 1, said selecting being performed in conjunction with computer modeling;

b) adding the potential antagonist or inhibitor to a glycoprotein synthesis assay in which the α1,2-mannosidase is a rate-limiting factor; and

c) detecting changes in glycoprotein carbohydrate synthesis; wherein a potential antagonist or inhibitor that inhibits the maturation of carbohydrate on newly formed glycoproteins and stabilizes misfolded glycoprotein is selected as a potential drug.

14. A method according to claim 13, wherein the antagonist or inhibitor is used to treat a genetic disease.

15. A method according to claim 14, wherein the genetic disease consists of cystic fibrosis or pulmonary emphysema, and wherein the glycoprotein consists of a mutant of cystic fibrosis transmembrane conductance regulator (CFTR) or α1-antitrypsin, respectively.

16. A nucleic acid encoding a catalytic domain of a class I α1,2-mannosidase enzyme having an amino acid sequence of SEQ ID NO:1.

17. A nucleic acid encoding a catalytic domain of a class I ER α1,2-mannosidase enzyme wherein arginine 273 is replaced by a leucine.

18. The nucleic acid of claim 16 wherein said α1,2-mannosidase enzyme converts $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ of a glycoprotein.

19. The nucleic acid of claim 18 wherein said α1,2-mannosidase enzyme specifically converts $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ isomer B of a glycoprotein according to the activity profile illustrated in Figure 8B.

20. An expression vector comprising the nucleic acid of claim 16 operatively associated with an expression control sequence.

21. A cell transfected or transformed with the expression vector of claim 20.

22. A class I α1,2-mannosidase enzyme having a catalytic domain according to the amino acid sequence of SEQ ID NO:1.

23. Use of the enzyme of claim 22 to modify carbohydrate structures on glycoproteins.

24. Use of the enzyme of claim 22 in the treatment of a genetic disease.

25. Use of the enzyme of claim 22, wherein the genetic disease is cystic fibrosis, pulmonary emphysema or lysosomal storage disease.

26. A crystal of a protein-carbohydrate complex comprising a catalytic domain of a class I α1,2-mannosidase enzyme complexed with 1-

deoxymannojirimycin inhibitor, wherein said crystal effectively diffracts X-rays at a resolution of 1.59 Angström.

27. The crystal of claim 26, wherein said 1-deoxymannojirimycin inhibitor binds to calcium and to essential acidic residues present within a barrel at an active site of said enzyme.

28. A crystal of a protein-carbohydrate complex comprising a catalytic domain of a class I α1,2-mannosidase enzyme complexed with a 1-deoxymannojirimycin inhibitor, wherein said crystal diffracts X-rays at 1.59 Anströms.

29. The crystal of claim 28 wherein said 1-deoxymannojirimycin inhibitor binds to a top portion of the C-terminal β-hairpin at an active site cavity.

30. Use of the crystal of claim 28 in molecular modeling of α1,2-mannosidase inhibitors.

31. Use of a three-dimensional structure of an α1,2-mannosidase of yeast, for deriving a second three-dimensional structure of an α1,2-mannosidase.

32. Use of a three-dimensional structure of an α1,2-mannosidase of yeast as claimed in claim 31 wherein said second three-dimensional structure of an α1,2-mannosidase is obtained by molecular replacement.

33. Use of the three-dimensional structure of the $\alpha 1,2$-mannosidase of claim 31 wherein said second three-dimensional structure is from a mammalian.

34. Use of the three-dimensional structure of the $\alpha 1,2$-mannosidase of claim 32 wherein the mammalian is a human.

35. A three dimensional structure of a protein-carbohydrate complex comprising a catalytic domain of a class I $\alpha 1,2$-mannosidase enzyme, said $\alpha 1,2$-mannosidase specifically converting $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B of a glycoprotein, said crystal effectively diffracting X-rays in a resolution range from 1.54 to 50 Angströms.

36. The three dimensional structure according to claim 1, wherein said class I $\alpha 1,2$-mannosidase enzyme consists of the endoplasmic reticulum class I $\alpha 1,2$-mannosidase enzyme.

37. The three dimensional structure according to claim 2, wherein said $\alpha 1,2$-mannosidase is derived from a yeast.

38. The three dimensional structure according to claim 3, wherein said yeast consists of *Saccharomyces cerevisiae*.

39. The three dimensional structure according to claim 4, wherein said catalytic domain comprises a

space group of $P3_121$ and a unit cell of dimensions of $\alpha=\beta=90.00$ and $\gamma=154.6$.

40. The three dimensional structure according to claim 5, wherein said catalytic domain comprises a barrel of seven pairs of helices ($\alpha\alpha_7$).

41. The three dimensional structure according to claim 6, wherein said pairs of helices consist of a first set of parallel helices inner-disposed in the barrel, and a second set of helices anti-parallel to the first set.

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/21769 A3

(54) Title: THREE-DIMENSIONAL STRUCTURE AND CRYSTAL OF A CLASS I α1,2-MANNOSIDASE, AND METHODS OF USE THEREOF

(57) Abstract: The present invention relates to a crystal and a three-dimensional structure for the endoplasmic reticulum α1,2-mannosidase enzyme family, useful for the identification of structural determinants responsible for the specificity of the family enzymes and for the development of specific inhibitors to stabilize abnormal glycoproteins in genetic diseases such as cystic fibrosis and pulmonary emphysema.

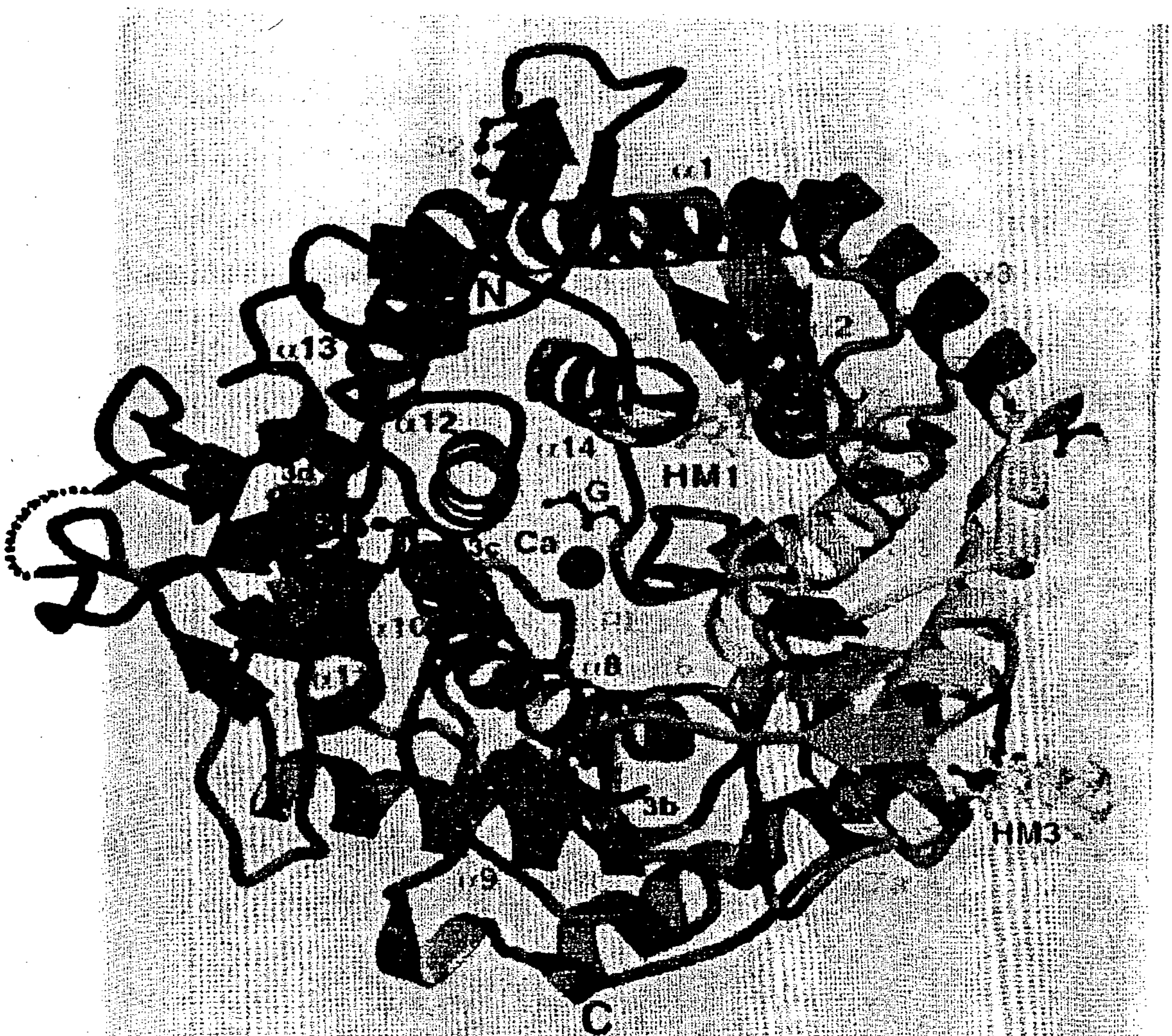
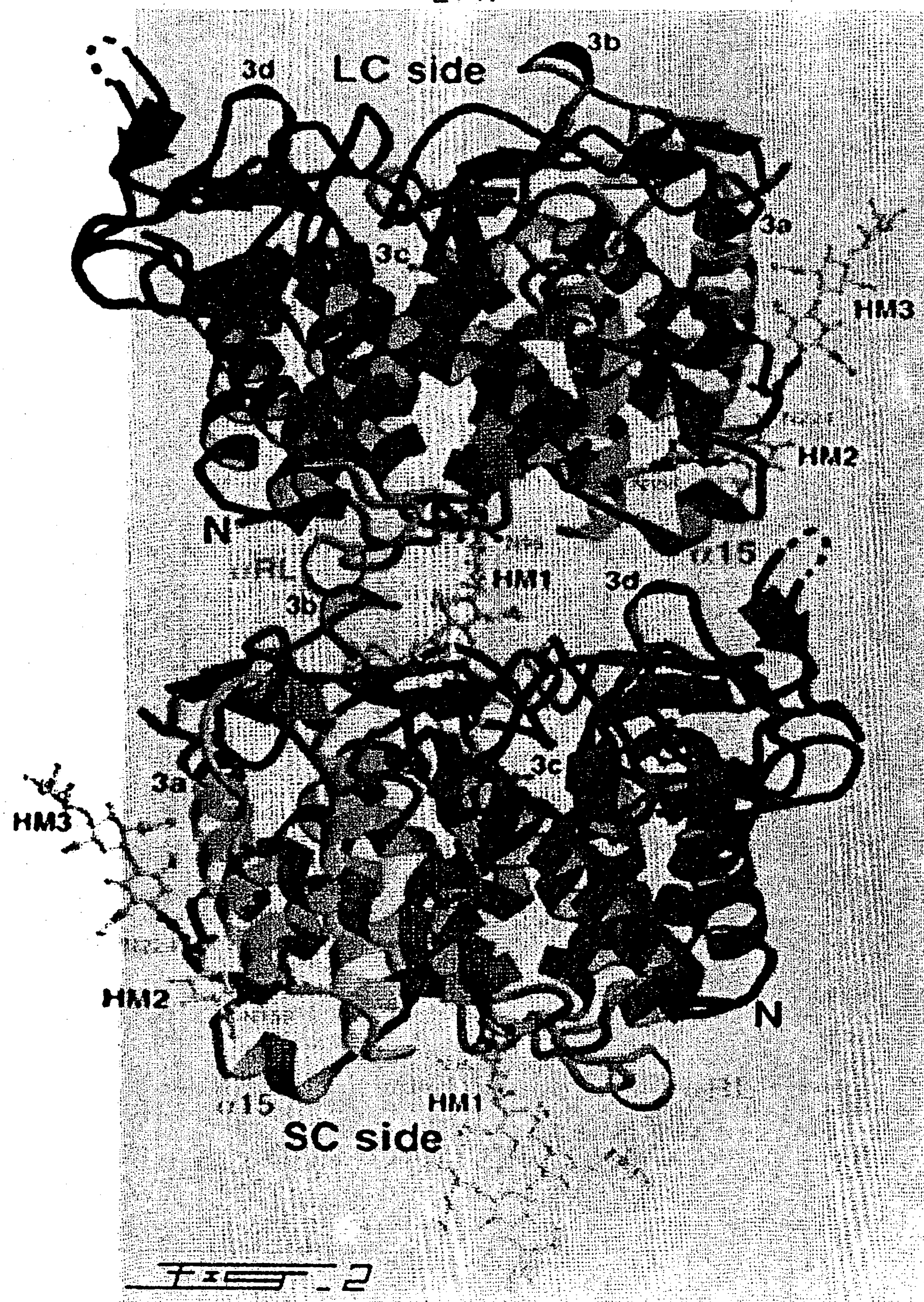


FIG. 1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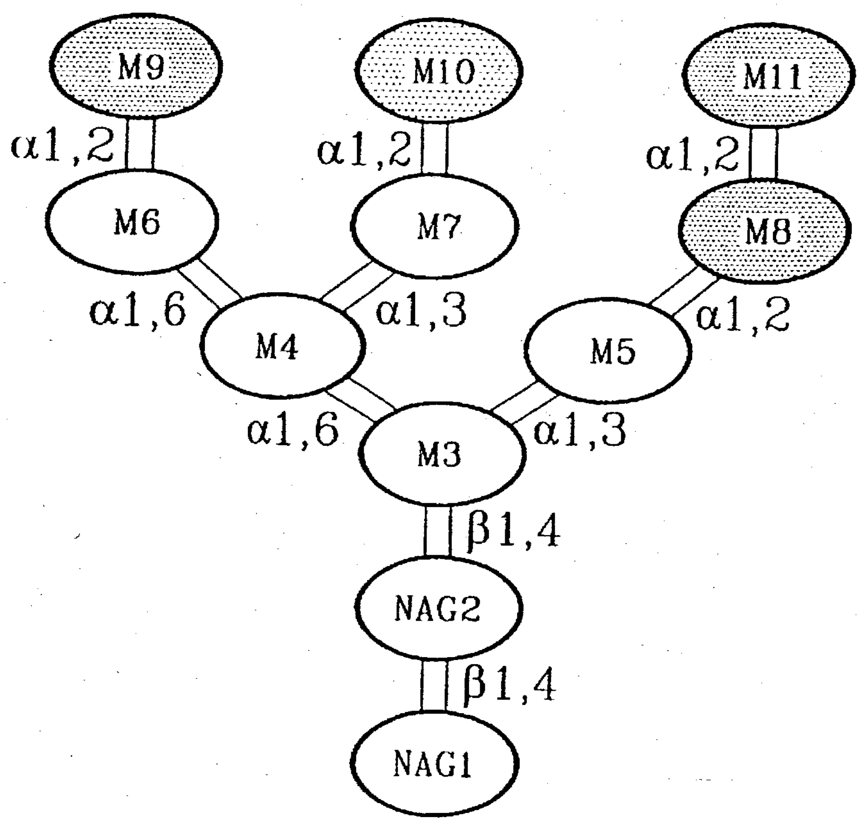
3b
3c
3a
HM3
HM2
N
RL
HM1
3d
α15
3b
3c
3a
HM3
HM2
N
α15
HM1
SC side

FIG. 2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M10
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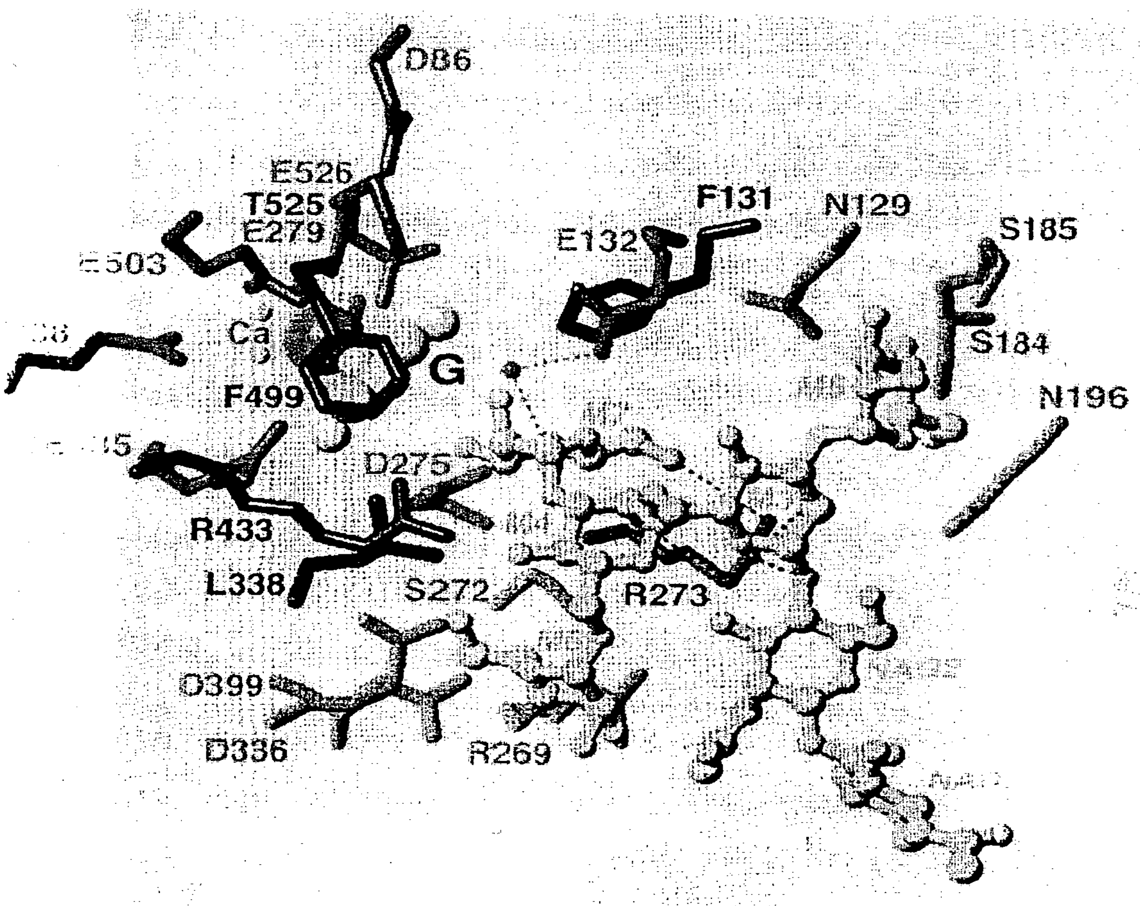
M4
M5
α1,6
α1,3
M3
β1,4
NAG2
β1,4
NAG1

FIG. 3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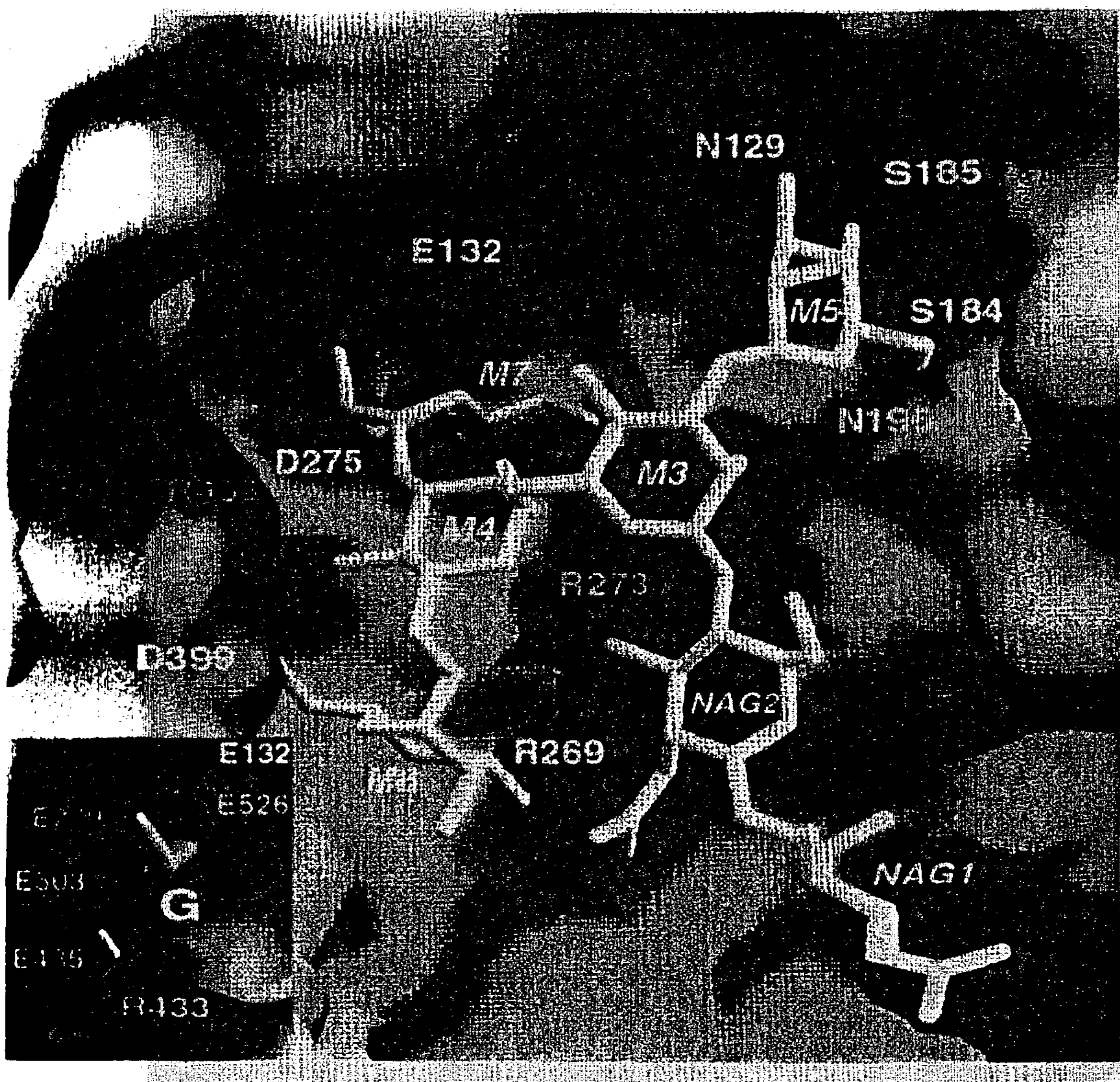
E526
T525
E279
E503
Ca
G
F499
F131
N129
E132
S185
S184
N196
D275
R433
L338
S272
R273
D399
D336
R269

FIG. 4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E132
M5
S184
M7
N190
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M3
M4
R273
D399
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E132
R269
E526
E503
G
R433
NAG1

FIG. 5

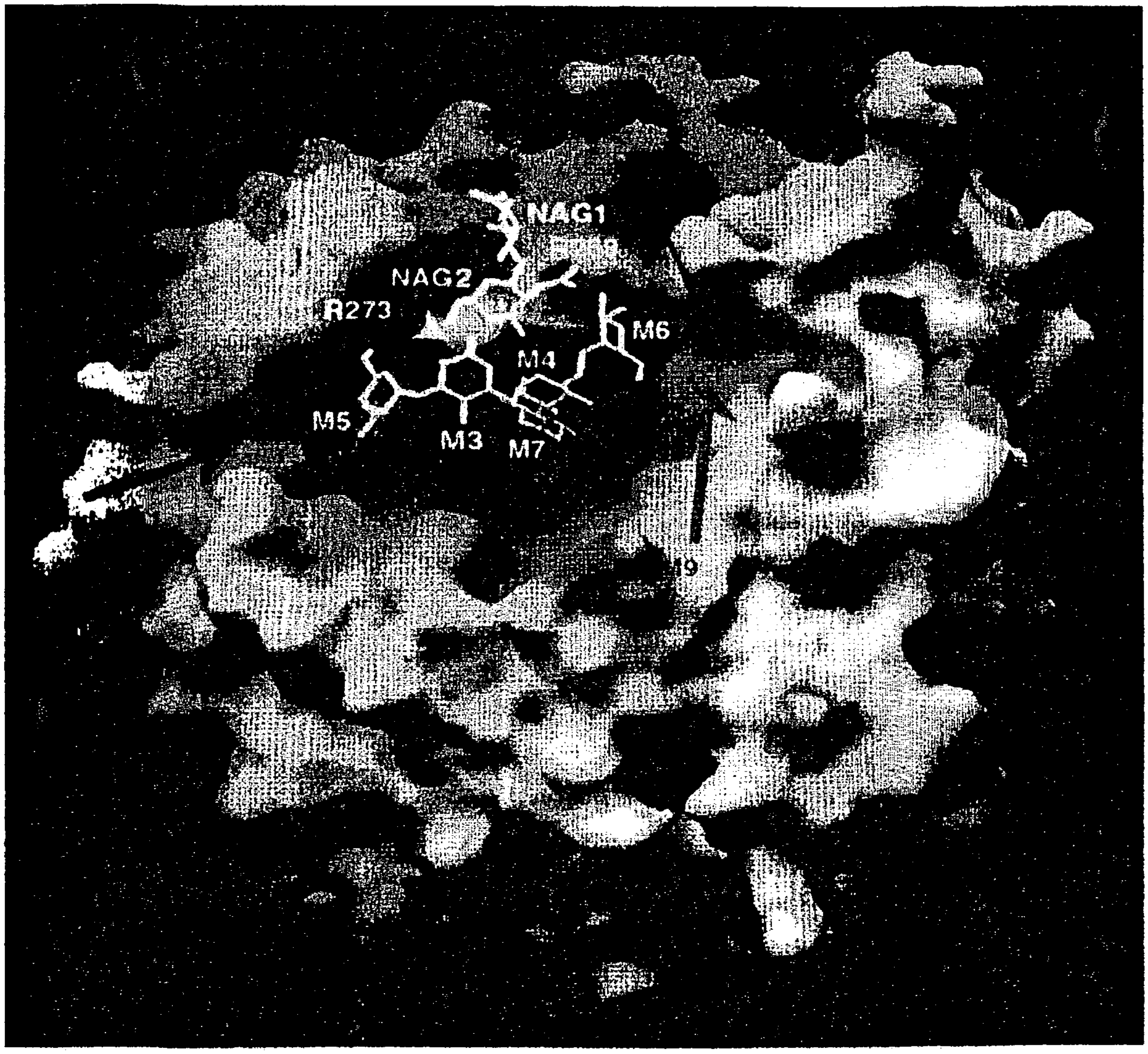

FIG. 6

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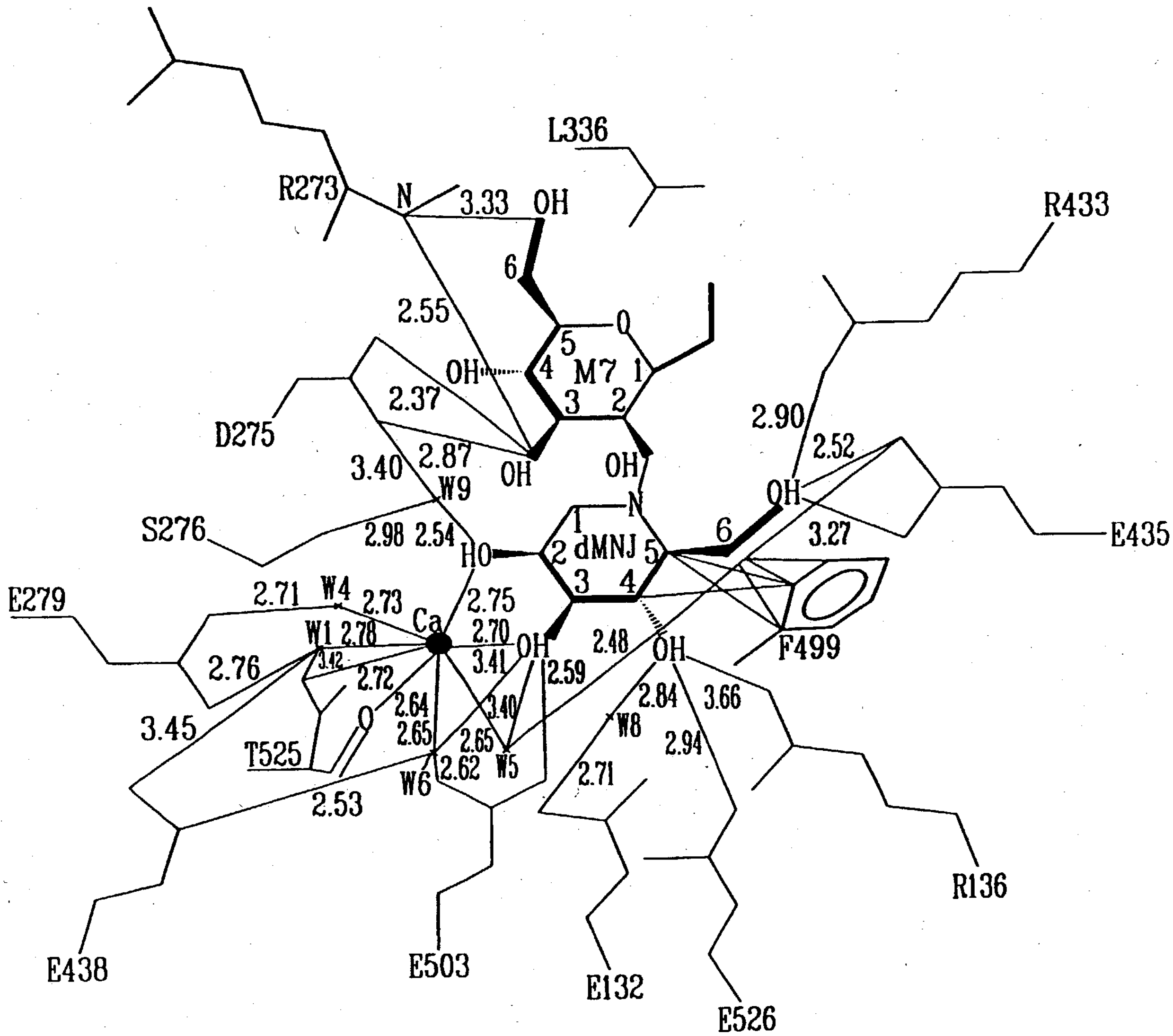
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3.33
OH
R433
6
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5
O
OH
4
M7
1
2.37
3
2
D275
2.90
2.52
2.87
OH
3.40
OH
W9
N
OH
S276
1
6
E435
2.98
2.54
HO
2
dMNJ
5
3.27
3
4
E279
2.71
W4
2.73
2.75
Ca
W1
2.78
2.70
OH
2.48
OH
F499
2.76
3.42
2.72
3.41
2.59
2.64
3.40
2.84
3.66
3.45
O
W8
T525
2.65
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W6
2.62
W5
2.71
2.53
R136
E438
E503
E132
E526

FIG. 7

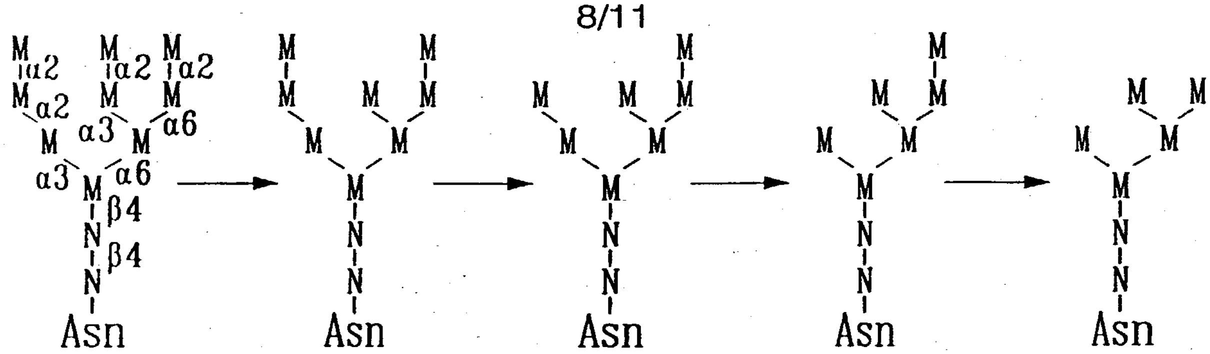

FIG. 8A

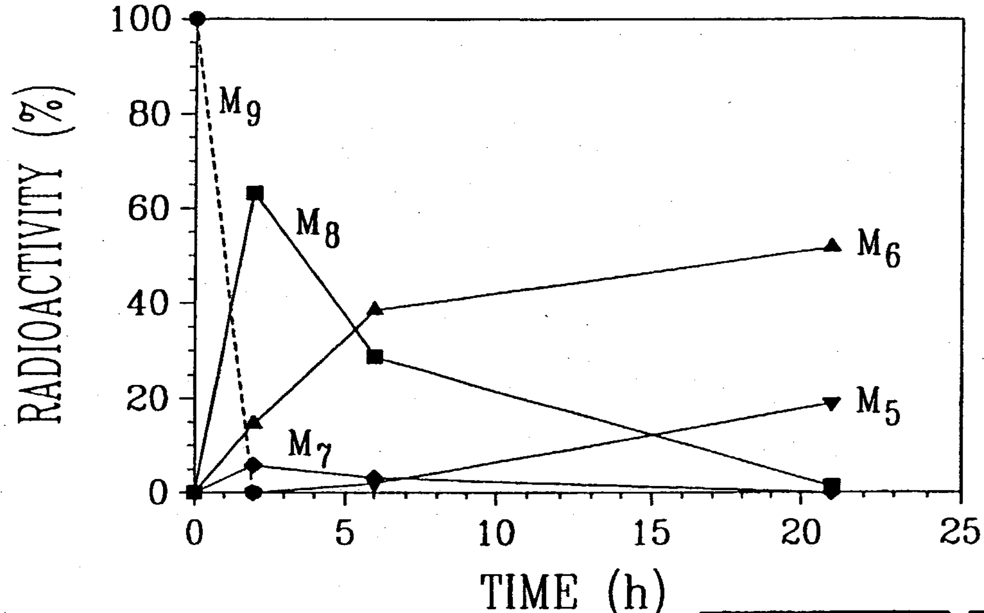

FIG. 8B

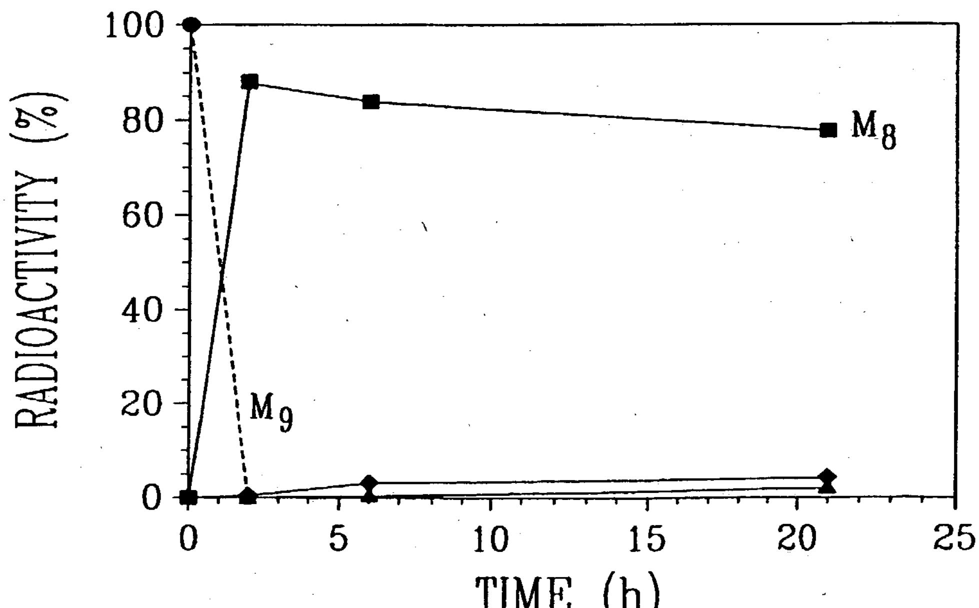

FIG. 8C

10/089000

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```
M K N S V G I S I A T I V A I I A A I Y Y V P W Y E H F E R K S P G A G E M R D   40
ATGAAGAACTCTGTCGGTATTTCAATTGCAACCATTGTTGCTATCATAGCAGCTATATACTATGTGCCATGGTACGAACACTTTGAGAGAAAGTCACCGGGGGCCGGAGAAATGAGAGAT   120
          *         *         *         *         *         *         *         *         *         *         *         *

R I E S M F L E S W R D Y S K H G W G Y D V Y G P I E H T S H N M P R G N Q P L   80
CGGATTGAAAGCATGTTCTTGGAATCGTGGAGAGACTATTCCAAGCATGGCTGGGGATACGATGTGTATGGACCTATTGAGCACACTTCCCATAATATGCCTCGTGGCAACCAGCCGTTA   240
          *         *         *         *         *         *         *         *         *         *         *         *

G W I I V D S V D T L M L M Y N S S T L Y K S E F E A E I Q R S E H W I N D V L   120
GGCTGGATTATCGTAGATTCAGTGGATACCTTGATGTTAATGTATAACTCCTCCACACTATACAAAAGTGAGTTCGAGGCAGAAATTCAGAGATCGGAGCATTGGATAAACGATGTTTTG   360
          *         *         *         *         *         *         *         *         *         *         *         *

D F D I D A E V N V F E T T I R M L G G L L S A Y H L S D V L E V G N K T V Y L   160
GATTTTGATATTGATGCCGAAGTGAATGTTTTTGAAACTACTATTAGAATGCTAGGTGGTTTATTATCCGCATATCATCTATCTGATGTTTTAGAAGTAGGTAATAAGACTGTCTACTTG   480
          *         *         *         *         *         *         *         *         *         *         *         *

N K A I D L G D R L A L A F L S T Q T G I P Y S S I N L H S G Q A V K N H A D G   200
AACAAAGCAATAGATTTGGGGGATAGGCTTGCTTTGGCGTTCTTATCCACTCAGACCGGAATTCCATACTCAAGTATAAACCTTCATAGTGGCCAAGCGGTTAAGAACCATGCAGATGGG   600
          *         *         *         *         *         *         *         *         *         *         *         *
```

FIG. 9

```
G  A  S  S  T  A  E  F  T  T  L  Q  M  E  F  K  Y  L  A  Y  L  T  G  N  R  T  Y  W  E  L  V  E  R  V  Y  E  P  L  Y  K     240
GGGGCATCTTCTACCGCAGAATTCACTACGCTACAAATGGAATTCAAATATCTGGCGTATTTGACAGGAAATCGTACTTATTGGAGCTGGTGGAGCGTGTTTACGAGCCATTATACAAA     720
         *         *         *         *         *         *         *         *         *         *         *         *

N  N  D  L  L  N  T  Y  D  G  L  V  P  I  Y  T  F  P  D  T  G  K  F  G  A  S  T  I  R  F  G  S  L  G  D  S  F  Y  E  Y     280
AATAACGATCTTCTAAATACCTACGATGGATTGGTTCCAATTTATACATTTCCAGATACTGGGAAGTTTGGTGCTTCGACTATCCGGTTCGGATCACTCGGGGATTCTTTTTATGAGTAT     840
         *         *         *         *         *         *         *         *         *         *         *         *

L  L  K  Q  Y  L  L  T  H  E  T  L  Y  Y  D  L  Y  R  K  S  M  E  G  M  K  K  H  L  L  A  Q  S  K  P  S  S  L  W  Y  I     320
TTACTAAAACAATATTTATTGACGCACGAAACACTTTATTATGATCTGTACAGAAAATCCATGGAAGGTATGAAAAAGCATTTATTAGCACAATCCAAACCCTCTTCTCTGTGGTACATT     960
         *         *         *         *         *         *         *         *         *         *         *         *

G  E  R  E  Q  G  L  H  G  Q  L  S  P  K  M  D  H  L  V  C  F  M  G  G  L  L  A  S  G  S  T  E  G  L  S  I  H  E  A  R     360
GGGGAAAGAGAACAAGGTCTACATGGACAACTTTCTCCTAAGATGGACCACCTCGTGTGCTTTATGGGGGGATTGTTAGCATCAGGCTCTACTGAGGGCCTTTCTATTCATGAAGCCCGA    1080
         *         *         *         *         *         *         *         *         *         *         *         *

R  R  P  F  F  S  L  S  L  E  R  K  S  D  W  D  L  A  K  G  I  T  D  T  C  Y  Q  M  Y  K  Q  S  S  S  G  L  A  P  E  I     400
AGACGTCCGTTTTTCTCTCTTTCCCTTGAAAGAAAAAGTGACTGGGATTTGGCTAAAGGGATAACTGACACATGTTATCAAATGTACAAGCAGTCTTCCTCGGGGCTTGCGCCTGAAATC    1200
         *         *         *         *         *         *         *         *         *         *         *         *
```

FIG. 9 (cont.)

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```
V  V  F  N  D  G  N  I  K  Q  D  G  W  W  R  S  S  V  G  D  F  F  V  K  P  L  D  R  H  N  L  Q  R  P  E  T  V  E  S  I      440
GTTGTCTTCAATGATGGAAACATAAAACAGGATGGTTGGTGGCGGTCGTCTGTGGGTGATTTTTTTGTTAAACCACTCGATAGGCACAACCTACAAAGACCAGAAACGGTGGAATCGATT   1320
          *         *         *         *         *         *         *         *         *         *         *         *

M  F  M  Y  H  L  S  H  D  H  K  Y  R  E  W  G  A  E  I  A  T  S  F  F  E  N  T  C  V  D  C  N  D  P  K  L  R  R  F  T      480
ATGTTCATGTATCATTTATCTCATGATCACAAATATCGTGAATGGGGGGCGGAAATCGCAACTAGCTTCTTTGAAAATACCTGTGTTGATTGTAATGACCCAAAATTAAGGCGGTTCACC   1440
          *         *         *         *         *         *         *         *         *         *         *         *

S  L  S  D  C  I  T  L  P  T  K  K  S  N  N  M  E  S  F  W  L  A  E  T  L  K  Y  L  Y  I  L  F  L  D  E  F  D  L  T  K      520
AGTTTAAGTGATTGTATCACGTTACCTACAAAGAAATCTAACAATATGGAAAGTTTCTGGTTGGCAGAGACTTTAAAGTATTTATATATATTGTTTTTAGACGAGTTTGATTTGACCAAA   1560
          *         *         *         *         *         *         *         *         *         *         *         *

V  V  F  N  T  E  A  H  P  F  P  V  L  D  E  E  I  L  K  S  Q  S  L  T  T  G  W  S  L                                        549
GTTGTTTTCAACACAGAAGCTCATCCTTTTCCAGTATTAGACGAAGAAATATTAAAATCGCAGTCTCTGACCACAGGTTGGTCGTTGTAG                                      1647
          *         *         *         *         *         *         *         *         *
```

FIG. 9 (cont.)

#7

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION

(37 CFR 1.63)

☐ Declaration Submitted With Initial Filing

☒ Declaration Submitted After Initial Filing (surcharge (37 CFR 1.16(a)) required

Attorney Docket No.:	SWA4338P0070US
First Named Inventor:	Annette Herscovics
COMPLETE IF KNOWN	
Application Number:	10/089,000
Filing Date:	March 22, 2002
Group Art Unit:	
Examiner Name:	

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Three Dimensional Structure and Crystal of a Class Iα1, 2-Mannosidase, and Methods of Use Thereof**, the specification of which:

☐ is attached hereto; or

☒ was filed on March 22, 2002 as Application Serial No. 10/089,000 and was amended on __________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information which is material to patentability as defined in 37 CFR. 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
CA00/01093	PCT	09/22/00	☐	☐	☒
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet attached hereto.

I hereby claim the benefit of any United States application(s) listed below.

Application Number(s)	Filing Date	
60/155,469	09/23/99	☐ Additional application numbers are listed on a supplemental priority data sheet attached hereto.

The undersigned hereby authorizes the U.S. attorney(s) or agent(s) named herein to accept and follow instructions from the assignee, if any, of the undersigned or from as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney(s) or agent(s) and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney(s) or agent(s) named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the following registered practitioner(s) identified by Customer No. 32116 to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, whose firm name, mailing address, telephone number, and facsimile number for this application are:

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Citicorp Center, Suite 3800
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Chicago, Illinois 60661-2511

Telephone (312) 876-1800
Facsimile (312) 876-2020

Customer Number (32116)
~~and/or Bar Code Label:~~

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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☐ A petition has been filed for this unsigned inventor.		

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Signature:		Date:
☐ A petition has been filed for this unsigned inventor.		

Name of Additional Inventor, if any:	Francois Vallee	
Citizenship:	FR/CA	
Residence:	490 Eglinton Avenue East, Apt. 405, Toronto, Ontario M4P 1M4 Canada	
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Signature:		Date:
☐ A petition has been filed for this unsigned inventor.		

Name of Additional Inventor, if any:	Francesco Lipari	
Citizenship:	Canada	
Residence:		
Post Office Address *(if different)*:		
Signature:		Date:
▢ A petition has been filed for this unsigned inventor.		

Name of Additional Inventor, if any:	Barry Sleno	
Citizenship:	Canada	
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Name of Additional Inventor, if any:	Francois Vallee	
Citizenship:	FR/CA	
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Name of Additional Inventor, if any:	Francesco Lipari
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Signature:	Date:
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Residence:	72 Meloche, Ste-Anne-de-Bellevue, Quebec H9X 3Z5 CA
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any: 5-00	Lynne P. Howell
Citizenship:	Canada
Residence:	10 Queens Quay West, Apt. 1704, Toronto, Ontario M5J 2R9 CA CAX
Post Office Address *(if different)*:	
Signature: P. Lynne Howell	Date: Dec 18 2002
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any:	Francois Vallee
Citizenship:	FR/CA
Residence:	490 Eglinton Avenue East, Apt. 405, Toronto, Ontario M4P 1M4 Canada
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any:	Francesco Lipari
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Residence:	8139 Page, LaSalle, Quebec H8P 3M3 CA
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any:	Barry Sleno
Citizenship:	Canada
Residence:	72 Meloche, Ste-Anne-de-Bellevue, Quebec H9X 3Z5 CA
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any:	Lynne P. Howell
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Post Office Address *(if different)*:	
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100

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Citizenship:	
Residence:	
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any:	
Citizenship:	
Residence:	
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

Name of Additional Inventor, if any:	
Citizenship:	
Residence:	
Post Office Address *(if different)*:	
Signature:	Date:
☐ A petition has been filed for this unsigned inventor.	

-44-

PTO/SB/122 (10-01)
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Application

Address to:
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Washington, D.C. 20231

Application Number	10/089,000
Filing Date	March 22, 2002
First Named Inventor	Herscovics et al
Art Unit	Unassigned
Examiner Name	Not Yet Known
Attorney Docket Number	SWA4338P0070US

Please change the Correspondence Address for the above-identified application to:

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OR

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Address	500 West Madison Street				
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Country	United States of America				
Telephone	312-876-1800	Fax	312-876-2020		

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I am the :

☐ Applicant/Inventor.

☐ Assignee of record of the entire interest.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☑ Attorney or Agent of record.

☐ Registered practitioner named in the application transmittal letter in an application without an executed oath or declaration. See 37 CFR 1.33(a)(1). Registration Number ____________

Typed or Printed Name	Martin L. Katz, Reg. No. 25,011
Signature	Martin L Katz
Date	June 6, 2002

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of one forms are submitted.